ROLE OF T-LYMPHOCYTES IN RADIATION-IMPAIRED WOUND HEALING

MICHAEL SCHÄFFER, CHRISTINA STÜLTEN, RICHARD VIEBAHN
Department of Surgery, University Hospital, Knappschaftskrankenhaus Bochum-Langendreer, Germany
Kierownik: prof. dr R. Viebahm

Radiation impairs healing, although the underlying mechanisms are not clearly defined. T-lymphocytes have been shown to be critical for wound healing. We hypothesized that radiation-impaired healing may affect different subtypes of T cells.

The aim of the study. We studied the effect of local electron irradiation on standard parameters of dermal wound healing in rats and correlated the outcome of healing with the expression of different lymphocyte subtypes in the wound.

Material and methods. Groups of 10 rats were irradiated using single dose 12 or 24 Gray electron radiation at the dorsal skin. Control rats were sham-irradiated. On day 5, a skin incision in the irradiated area was performed and polyvinyl alcohol sponges were inserted subcutaneously. Rats were sacrificed 10 days later to determine the wound breaking strength and reparative collagen deposition. Blood lymphocytes were analyzed by FACS. Immunohistochemistry was performed on wound sections.

Results. Irradiation significantly reduced wound collagen deposition and wound breaking strength (p <0.05), leading to a 78% reduction in collagen deposition and 47% reduction in breaking strength in 24 Gy animals. Blood lymphocytes were not affected by electron-irradiation, suggesting that the wound was not affected by radiation-induced systemic effects. Impaired healing was reflected, however, in increased expression of wound CD8 cells and decreased expression of CD25 (IL-2 receptor) (p<0.01). No effect was seen on wound CD4 cells. In addition, the ratio of CD4/CD8 was significantly decreased (p<0.05).

Conclusions. Radiation-impaired healing is reflected in impaired expression of wound lymphocyte subtypes. Altered lymphocyte subtypes may affect the outcome of healing in irradiated wounds.

Key words: wound healing, radiation, lymphocyte, CD4, CD8, CD25

Injury activates a cascade of local and systemic immune reactions. An intact cellular immune response is essential for a normal outcome of tissue repair (1). Lymphocytes represent a critical part in the cellular host immune response. Global T-cell depletion has been shown to cause a marked diminution in the wound mechanical strength and wound collagen content (2). Antibody-induced depletion of the CD8+ subset has been found to cause a marked enhancement of wound healing (3). These findings suggest a possible role for the CD8+ cells in the overall down-regulation of wound healing activity. It might be expected that the CD4+ T-helper cells would promote such activity. Depletion of this subset by an anti-L3T4 antibody, however, was shown to have no effect on either wound mechanical strength or wound collagen formation (3). Therefore, an incompletely characterized lymphocyte population bearing the all T-cell antigenic marker Thy-1.2, but not the mentioned determinant for CD4 or CD8, seems to be responsible for the promotion of wound healing, since its depletion impairs wound healing.

Increasing experience with radiation therapy has extended the limits of surgical oncology. Radiation, however, impairs wound healing.

Due to their accelerated cell turnover, fresh wounds are susceptible to growth inhibition by
preoperative or immediate postoperative irradiation. The mechanisms leading to diminished wound repair, however, are poorly understood (4). Electron irradiation has become readily available for use in radiotherapy. This procedure produces large differences in depth-dose distribution (when compared with photon irradiation) thus enabling the delivery of a maximal dose to skin and subcutaneous tissue (5). There is a high dose of skin entry and a rapid fall in energy at increased depth, minimizing systemic radiation effects.

We studied the effect of local electron irradiation on standard parameters of dermal wound healing in rats and correlated the outcome of healing with the expression of different lymphocyte subtypes in the wound.

MATERIAL AND METHODS

Thirty male Sprague Dawley rats (Charles River, Sulzfeld, Germany) weighing between 220 and 250 g were allocated at random to sham-irradiated controls (n = 10) and two irradiated experimental groups (12 Gy and 24 Gy, n = 10 respectively). Animals were caged in groups of two animals and allowed one week to acclimate. The animals were fed a complete pelleted laboratory chow and had access to tap water ad libitum. All procedures were reviewed and approved by the local Institutional Animal Care and Use Committee.

Irradiation procedure

All animals were anaesthetized by ketamine and xylazine (100 and 15 mg/kg body weight intraperitoneally, respectively). Rats, in groups of three, were placed ventral surface downwards into a custom-made Plexiglas container which permitted the simultaneous irradiation of three animals. Animals were placed in a 45° angle (45° left body part up) to irradiate the left side of the back of the animals. To calibrate the electron radiation and to determine the central axis depth ionization curve, readings were taken at a constant focus-to-chamber distance for increasing layers of Plexiglas, and the subsequent radiographic recordings were analyzed by microdensitometry. Prior to irradiation, the output of the accelerator was measured at the position occupied by the central animal, and these data were used to determine the exposure time and dose. Irradiation was performed with 4 MeV electrons, using a fixed target-to-sour-
scar. Breaking strength was tested on a tensiometer (Zwick, Ulm, Germany) (8), using a constant speed of 50 mm/min. The force at which the strips broke was measured in Newton (N). Strip 7 was used for histology.

Hydroxyproline

The two most cephalad-placed polyvinyl alcohol sponges were harvested, cleared of surrounding tissue, and stored at -80°C for subsequent assay of hydroxyproline content, an index of reparative collagen deposition, using a calorimetrical method (8, 9). The values of the two sponges were averaged for each animal. The remaining sponges were retrieved for histology and immunohistochemistry.

Leukocyte analysis in blood

Total leukocyte count (WBC) and differential leukocyte count were performed in blood obtained by cardiac puncture at the day of sacrifice. In addition, peripheral blood mononuclear cells were analyzed by direct immunofluorescence (FacsSort, Becton Dickinson, Heidelberg, Germany) for CD4 and CD8 antigen expression, using direct fluorescence-marked antibodies (mouse anti-rat CD4-phycocerythrin, mouse anti-rat CD8-flourescein, Pharmingen, Hamburg, Germany). Data was acquired with Cell Quest software (Becton Dickinson, Heidelberg, Germany).

Leukocyte analysis in the wound

Wound sections and sections of subcutaneous implanted sponges, harvested 10 days post-wounding, were stained by standard hematoxylin and eosine for morphological assessment. Immunohistochemical staining was used to identify the CD4, CD8, and CD25 in sponge infiltrating cells and wound sections.

Wound sections and sponges harvested 10 days post-wounding were fixed in 10% neutral buffered formalin at 4°C for 24 hours, paraffin embedded, and sectioned (4 µm). Following deparaffinization and endogenous peroxidase inactivation, nonspecific binding was blocked (horse serum), and sections were incubated overnight at 4°C with monoclonal mouse anti-rat CD4 antibody (Serotec Ltd., Kidlington, Oxford, UK), monoclonal mouse anti-rat CD8 antibody (PharMingen, Hamburg, Germany), or monoclonal mouse anti-rat CD25 antibody (PharMingen, Hamburg, Germany). Subsequently, slides were washed and incubated for 30 min at 37°C with secondary antibody (biotinylated horse anti-mouse IgG, Linaris, Wertheim-Bettingen, Germany) for CD4 and CD8, or monoclonal mouse anti-rat CD25 antibody (PharMingen, Hamburg, Germany). Subsequently, slides were immersed with streptavidin biotin complex (No. K-0377, Dako) for 30 min at room temperature. Finally, slides were counterstained with Mayer’s hematoxylin. A computerized Quantimet 500 Image Analyzer (Leica, Jena, Germany), using three random fields of a minimum of 100 cells each, was used for quantification of positive staining cells.

Fig. 1. Effect of irradiation on (A) wound-breaking strength (WBS; N = Newton) of incisional wounds and (B) hydroxyproline (OHP) content, an index of wound collagen accumulation, in subcutaneously implanted polyvinyl alcohol sponges 10 days post-wounding. Animals were irradiated 5 days pre-wounding with 12 and 24 Gy electron radiation. Control animals were sham-irradiated. n = 10, *p<0.05 versus control, †p<0.01 versus control.
Statistical analysis

All data are reported as means ± standard error of the mean (SEM). Statistical analysis was performed by applying ANOVA followed by Scheffé’s test using the StatView II statistical package (Abacus Concepts, Berkeley, CA) on a Macintosh computer. Statistical significance was achieved at p<0.05.

RESULTS

The radiation treatment and wounding procedure were equally well tolerated by all animals, as reflected by equal weight gain of all groups (Control: 26 ± 2 %, 12 Gy: 29±3%, 24 Gy: 21±3%, p = 0.70). Total leukocyte count, differential leukocyte count, and CD4/CD8 ratio were similar among the groups (tab. 1). This suggests that electron irradiation had little systemic effect.

Irradiation significantly reduced wound collagen deposition and wound breaking strength (p<0.05), leading to a 78% reduction of collagen deposition and 47% reduction of breaking strength in 24 Gy animals. There were no wound infections as assessed clinically and by testing wound fluid for sterility.

Ten days post-wounding, histology (hematoxylin-eosine) showed no major differences between the skin wound sections and the sponge sections of the different groups. In the wounds there was a similar infiltrate of inflammatory cells. Epithelialization of all skin wounds was found to be completed. Also, within each group the cellular infiltrate of the skin wound resembled that of the subcutaneously implanted sponges.

Immunohistochemical staining, performed on sponge sections from controls and animals irradiated with 24 Gy electron radiation, revealed different lymphocyte subtypes throughout the wound. Figure 2 shows an example of CD4 and CD8 expression in wounds of animals irradiated with 24 Gy. Computerized quantification of positive staining cells demonstrated that impaired healing was paralleled by an increased expression of wound CD8+ cells and a decreased expression of CD25 (p<0.01). No effect was seen on wound CD4+ cells. In addition, the ratio of CD8/CD4 was significantly increased (p<0.05) (tab. 2).

DISCUSSION

Electron irradiation impairs wound healing in rats, and this is reflected by an increased expression of CD8+ lymphocytes, a decrease in CD4/CD8 ratio, and a diminished expression of CD25 in the wound.

Clinically and experimentally, radiation has been shown to impair wound repair, irrespective of inclusion (radiation to hemibody or total body) or exclusion (radiation to skin alone) of the hematopoietic system in the field of irradiation (10, 11, 12). It appears that wound mechanical strength is similarly affected by radiation applied at any time point between two months preoperatively and five days postoperatively (11, 13). A maximum early acute skin reaction following a single dose exposure to 12 Gy, however, appears at about one week post-irradiation (6, 7). For this reason, rats were irradiated within this time frame before wounding. Radiation affects different phases of healing, including inflammation, proliferation, and maturation. Diminished phagocytosis, superoxide production, and Mac-1 expression by neutrophils may explain increased susceptibility to infection (14). Following irradiation, proliferation of endothelial cells and fibroblasts, as well as collagen production and fibroblast-mediated contraction, are reduced (15, 16), leading to impaired mechanical strength and diminished wound collagen deposition. This may

Table 1. Total leukocyte count (WBC), differential leukocyte count, and C4/CD8 ratio determined in the blood ten days after wounding. Animals were sham-irradiated (control) or irradiated with 12 or 24 Gy five days prior to wounding. Eosinophils and Basophils were found to be <1% in all animals. No significant differences were found between the groups (n = 10, F-Test, ANOVA)

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBC (x 10^3/μl)</th>
<th>Neutrophil (%)</th>
<th>Lymphocyte (%)</th>
<th>Monocyte (%)</th>
<th>CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.5 ± 0.8</td>
<td>25.2 ± 0.8</td>
<td>68.2 ± 1.1</td>
<td>6.5 ± 0.3</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>12 Gy</td>
<td>11.2 ± 0.6</td>
<td>25.5 ± 2</td>
<td>68.3 ± 3.7</td>
<td>6.3 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>24 Gy</td>
<td>13 ± 1.2</td>
<td>26.2 ± 1.3</td>
<td>66.3 ± 2.2</td>
<td>6.9 ± 0.4</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>F-Test</td>
<td>p = 0.34</td>
<td>p = 0.89</td>
<td>p = 0.82</td>
<td>p = 0.48</td>
<td>p = 0.34</td>
</tr>
</tbody>
</table>
be due, at least in part, to radiation-induced DNA damage, leading to altered cell function. In the present study, no major histological differences in inflammatory cell infiltrate were found between the groups 10 days post-wounding. Cellular activity and/or the composition of the different kinds of cells, rather than the total number of infiltrating cells, may be responsible for radiation-impaired healing. Differences in cellular infiltrate between irradiated and non-irradiated wounds at earlier time points post-wounding, however, can not be excluded by our experiments.

Histologically, the sponges did not exhibit a more pronounced inflammatory cell infiltrate than the wound sections, suggesting that there is only a small amount of sponge-induced foreign body reaction. Polyvinyl alcohol sponges allow repair-promoting cells to infiltrate, creating a well-defined new granulation tissue. Irradiation affected both skin and sponge granulation tissue. Also, impaired skin wound mechanical strength in irradiated animals was paralleled by decreased sponge collagen deposition.

Global T-cell depletion, using the 3OH12 monoclonal antibody against the Thy-1.2 antigen, causes a depression of wound breaking strength and wound collagen content. Anti-Lyt 2 antibody-induced depletion of the CD8+ subset causes marked improvement of wound healing (2, 3). Thus, in turn, it is conceivable that an increase of CD8+ cells, as found in our experiments, will lead to diminished wound repair. Further support for these findings is provided by work in congenitally athymic nude mice (17, 18, 19). These animals have a profoundly impaired T-cell dependent system and display significantly enhanced wound breaking strength and collagen deposition in response to injury when compared to normal thymus-bearing animals. T-cell reconstitution of nude mice with syngenic splenic T-lymphocytes resulted in significant decreases in wound mechanical strength and collagen formation, similar to the values observed in normal euthymic controls.

Table 2. Percentage of cells staining positive for expression of CD4, CD8, CD4/CD8, and CD25 in wounds 10 days after wounding. Animals were sham-irradiated (control) or irradiated with 12 or 24 Gy five days pre-wounding

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>CD4/CD8 (%)</th>
<th>CD25 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63 ± 7,9</td>
<td>4,1 ± 1,5</td>
<td>15,4 ± 3,3</td>
<td>8,2 ± 2,2</td>
</tr>
<tr>
<td>12 Gy</td>
<td>55 ± 7,9</td>
<td>9,2 ± 1,3*</td>
<td>6 ± 2,4*</td>
<td>5,6 ± 1,9</td>
</tr>
<tr>
<td>24 Gy</td>
<td>61,3 ± 3,9</td>
<td>14 ± 2,9*</td>
<td>4,4 ± 1,3*</td>
<td>3,5 ± 1,2*</td>
</tr>
</tbody>
</table>

n = 10, *p < 0,05 w porównaniu z grupą kontrolną / versus control, test Scheffégo / Scheffé-Test

Fig. 2. Immunohistochemical staining of wound sponge sections for CD4 and CD8. Sections were stained with monoclonal mouse anti-rat CD4 and CD8 antibody as described in “Material and methods”. A – CD4 expression in wound section from animal irradiated with 24 Gy electron radiation; B – CD8 expression in wound section from animal irradiated with 24 Gy electron radiation. Magnification 360x
This supports the hypothesis that a subset of T-cells normally plays a down-regulatory role in wound healing.

Some information regarding the role of T-lymphocytes in wound repair has been obtained from in vivo studies which used lymphotrophic or lympholytic agents (20). The parenteral or enteral administration of agents known to enhance thymic and T-lymphocyte function, such as growth hormone (21), vitamin A (22), or arginine (23), leads to increases in wound mechanical strength and collagen deposition. Agents which suppress T-lymphocyte function, such as steroids and retinoic acid, markedly impair wound healing (22). In addition, it has been demonstrated that systemic treatment with ciclosporin and tacrolimus, both immunosuppressive drugs known to inhibit T-cell proliferation and IL-2 synthesis by interfering with intracellular calcineurin, leads to impaired wound healing in rats (24, 25). Furthermore in our experiments, CD25 (IL2 receptor alpha chain), expressed on antigen-activated T-cells, has been found to be suppressed in irradiated wounds, indicating a substantial alteration of the state of activation of lymphocytes present at the wound site.

Modification of T-lymphocyte function by adult thymectomy, which prevents the induction of CD8+ T-cells, causes an increase in wound mechanical strength (26). This effect can be reversed by intraperitoneal placement of autologous thymic grafts in thymectomised rats. Conversely, administration of thymic hormones, such as thymulin or thymopentin, results in impaired wound healing as assessed by wound mechanical strength and wound collagen deposition (20). These data suggest that the thymus has an inhibitory effect on healing.

The T-cell contribution to the healing process is mediated in part by the release of soluble factors (lymphokines). A variety of in vitro and in vivo studies have tried to elucidate the role of lymphokines in fibroblast regulation and in vivo tissue repair.

Many lymphokines are capable of modulating fibroblast replication, migration, or collagen synthesis in vitro. Some, such as transforming growth factor-β (TGF-β) or interferon-γ (IFN-γ), are well characterized, while many other mediators which can modulate in vitro activity of fibroblasts or keratinocytes remain incompletely characterized (27). Both inhibitory and stimulatory lymphokines for all aspects of fibroblast function have been described. While the in vivo regulation of wound healing most likely consists of a complex multifactorial interaction between various cytokines and growth factors including the T cell-derived lymphokines, it remains unclear how these factors act in temporal sequence and in concert. In athymic nude mice exhibiting an impaired T-cell dependent system and enhanced wound repair, increased expression of basic fibroblast growth factor and insulin-like growth factor-1 was found (19). Clinically, in pilonidal sinus wounds, the CD4/CD8 ratio fell throughout the healing process. This was accompanied by an increase of CD8+ cells at the time of wound closure, suggesting a CD8+ lymphocyte-mediated downregulation of healing (28).

In conclusion, radiation-impaired healing is reflected in impaired expression of wound lymphocyte subtypes. Altered lymphocyte subtypes may affect the outcome of healing in irradiated wounds. In the wound it remains unclear whether lymphocytes act mainly through surface antigens or through the release of active mediators (lymphokines) (29). A change of mediator expression may interfere with normal cell-to-cell interaction of other inflammatory and mesenchymal cells, causing impaired wound repair. The exact mechanisms of radiation-induced impaired healing need to be further elucidated.

REFERENCES


Received: 28.01.2007 r.
Address correspondence: Chirurgische Universitätsklinik Knappschaftskrankenhaus
In der Schornau 23-25, 44892 Bochum-Langendreer, Germany